TECHNICAL MANUSCRIPT 141

BOTULINUM TOXOIDS

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ABSTRACT

Procedures have been developed for preparing purified aluminum-phosphate-adsorbed, univalent, bivalent, and pentavalent botulinum toxoids to immunize man. All preparations were well tolerated and elicited satisfactory antitoxin responses in man, whether administered as a single antigen or in combination. Four separate pentavalent Type ABCDE toxoids produced immune responses to each antigen in a considerable proportion of individuals following an initial series of three injections. A booster injection administered one year after the initial injection markedly increased the antitoxin titers and measurable antitoxin titers were found in 86 to 100 per cent of the individuals immunized. The toxoids were antigenic for mice, guinea pigs, and rabbits. In guinea pigs the toxoids afforded a high level of resistance to challenge with toxins administered by various routes.

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I. INTRODUCTION

Botulism in human beings is a rare disease. As a result there exists no need for immunizing large numbers of persons against it. It is useful, however, to provide immunization for laboratory workers engaged in studies and research on botulinum toxins. The disease occurs world wide[1] though its geographic distribution is irregular.[2] In some areas it has been a serious problem in domestic animals and in water fowl. The prevalence among sheep and cattle in Western Australia and among cattle in South Africa, which fed on toxic carrion, and in domestic mink, fed on improperly handled mammalian or fish feed, warranted generalized immunization of these animals against this disease.

Much has been published on the preparation of botulinum toxoids since the first reports by Weinburg and Coy in 1924,[3] and the antigenicity of botulinum toxoids prepared in various ways has been demonstrated in experimental animals by a number of early investigators.[4-9] The experimental immunization of man with toxoid was reported in 1934 and 1936 by Velikanov.[10,11] Melnik and Starobinetz[12] in 1936 showed that an alum-precipitated toxoid produced antitoxin in guinea pigs. Preparation of an effective Type C toxoid for the control of botulism in sheep and cattle in Australia was described by Bennetts and Hall[13] in 1938.

Mason,[14] Sterne,[15] and their associates in South Africa demonstrated that mass immunization of cattle with combined Types C and D formal toxoids effectively and economically reduced cattle losses due to botulism. Research was intensified during World War II, and methods for the preparation of alum-precipitated Types A and B toxoids of high antigenic value in experimental animals and man were developed by Nigg and associates[16,17] in the United States and by Rice et al[18-20] in Canada. Prevot and Brygoo[21] produced Type C antitoxin in man by immunization with toxoid prepared from toxin extracted from bacterial cells. Methods for the preparation of Type C toxoid for the control of botulism in domestic mink[22-32] and game birds[33-38] have also been described. Barron and Reed[37] presented a systematic study of methods for preparing crude alum-precipitated Type E toxoid. Crude Type E and Type F toxoids have been prepared for the immunization of animals and the preparation of antitoxins.[38-50]

Although the bivalent Type AB toxoid developed and produced at Fort Detrick during World War II produced satisfactory immunity in man[51] as measured by serum antitoxin concentration, undesirable local and systemic reactions to injections of the relatively crude antigens were encountered. Subsequently, procedures for preparing a purified polyvalent toxoid of improved clinical acceptability were investigated in our laboratories. It is my purpose today to discuss the development of the purified botulinum toxoids and their evaluation in man and laboratory animals. The information to be presented today represents a summation of published investigations carried out in our laboratories by Duff,[52-54] Wright,[55] Gordon,[58] Flock,[57-59] Cardella,[60-63] and our associates. A brief outline of the methods employed in preparing the purified toxins and toxoids will be presented first.

Following this, data obtained on the assessment in man of univalent, bivalent, and pentavalent preparations will be presented. The concluding section of this report will present studies on the efficacy of toxoids, based on development of antitoxin titer and resistance afforded actively immunized animals to challenge with toxin by various routes.

II. METHODS OF PREPARATION

High-potency Types A, B, C, D, and E toxins were produced by growing highly toxigenic strains of Clostridium botulinum in culture media that were composed of commercially available constituents. Production methods could be applied readily on a scale suitable for routine production of a biological. Toxicity of Type E toxin was increased 50- to 100-fold by treatment with trypsin. The activation procedure allowed attainment of culture toxicities of the same order of magnitude as those obtained with Types A, B, C, and D. These results provide the explanation for the low apparent toxicity of Type E cultures, an observation that had been difficult to understand in view of the high mortality associated with outbreaks of Type E botulism. The five toxins were purified by chemical methods that included an initial precipitation, extraction with calcium chloride, one or two reprecipitations, and subsequent resolution into an appropriate buffer system. Specific activity of the purified toxins ranged from 40×10^6 mouse intraperitoneal LD_{50} ($MIPLD_{50}$) per milligram of total nitrogen with the Type C to approximately 500×10^6 $MIPLD_{50}$ per milligram of N with the Type D toxin. Ultracentrifugal analysis showed that the Types A and B sedimented as a single boundary. With Types, C, D, and E, the major sedimenting boundary represented 80 to 90 per cent of the protein concentration. The toxins were sterilized by filtration and converted to toxoids by incubation in the presence of formalin. Detoxification reactions deviated markedly from first-order behavior.

Toxin preparations were usually detoxified in the presence of 0.6 per cent formalin at 35° to 37°C and pH 5.5 to 6.5 in 15 to 25 days. Although the toxins were initially more toxic for mice than for guinea pigs, detoxification for mice occurred more rapidly than detoxification for guinea pigs. Fluid toxoids were readily absorbed on aluminum phosphate prepared as described by Holt[54] for diphtheria toxoids. Absorbed toxoids were prepared so that one milliliter of final toxoid contained 7 milligrams of adjuvant. Thimerosal in a concentration of 0.01 per cent was used as preservative. Gentle shaking for 18 to 24 hours was sufficient for adsorption of the toxoids. Safety testing included tests for the presence of toxin and standard sterility tests.

Procedures developed in our laboratories at Fort Detrick were subsequently adapted for large-scale production by Parke, Davis and Company under the direction of Dr. Henry B. Devlin. Univalent and polyvalent preparations of adsorbed toxoid were produced for experimental use. Clinical trials with the toxoids have been completed. Toxoids were administrated in either 0.25- or 0.5-milliliter amounts by deep subcutaneous inoculation in the deltoid region. The purified toxoids were well tolerated and were considered satisfactory clinically. Considerably less than one per cent of those immunized have shown a mild local reaction, a transient nodule of varying size that persisted for approximately two weeks.

Individual antigenic responses to botulinum toxoids were determined by serum neutralization titrations in mice, using the univalent Porton* (British) antitoxins as primary reference standards. The Porton antitoxins were established as International Standards at the fifteenth Session of the Expert Committee on Biological Standardization World Health Organization, Geneva, December 1962.[88] Purified toxins diluted with two parts glycerine and stored at minus 20°C were standardized against the reference antitoxins.

The units of antitoxin used for the standardization of the glycerinated toxins are presented in Table I. The neutralizing capacities of the units of the five types of antitoxin were different, but at the level of standardization each antitoxin neutralized approximately 10 LD_{50} of its homologous toxin.

TABLE I. UNITS OF ANTITOXIN USED FOR STANDARDIZATION OF TOXIN

Type	Units of Porton Antitoxin Per Milliliter
A	0.02
B	0.005
C	0.02
D	0.16
E	0.0125

* Microbiological Research Establishment, Porton, England.

In preliminary experiments, guinea pigs immunized with univalent toxoids and challenged intraperitoneally withstood 1 x 10^5 to 1 x 10^6 MIPLD$_{50}$'s of homologous toxin; the antitoxin titers for each type at the time of challenge were approximately two times the lowest measurable titer. The lowest measurable titers were, therefore, though somewhat arbitrarily, selected as satisfactory antitoxin levels.

Serum antitoxin titers of individuals who had received botulinum toxoids were determined at various intervals after primary immunization and again after booster immunization. Efficacy of the toxoids is generally expressed in terms of percentages of individuals developing these or greater titers.

III. ASSESSMENT IN MAN

The first studies in man were carried out with the univalent Type A toxoid containing 10.2 Lf*/ml. Three schedules of immunization were investigated: 0, 2, 4, and 6 weeks; 0, 2, and 4 weeks; and 0 and 8 weeks. The groups consisted of 11 to 35 persons. A single injection produced a measurable quantity of antitoxin in a few individuals. Although the 8-week injection on the 0-8 schedule appeared to have some boostering effect, the 0-2-4-6 schedule showed the highest number of persons with measurable titers at 12 weeks. Toxoids containing 1.7 and 0.34 Lf/ml did not appear to be as satisfactory for rapid immunization as the 10.2 Lf toxoid. Booster injections consisting of 0.25 milliliter of the 10.2 Lf toxoid were given to all individuals five to seven months after the initial injection of toxoid. Satisfactory antitoxin levels were present in all persons one month after the booster, irrespective of the primary immunization schedule. Although the reduced Lf toxoids exhibited a high booster response, the immunization with the 10.2 Lf toxoid on a 0-2-4-6 week schedule appeared to be most satisfactory for rapid immunization. Available data indicated that with the 10.2 Lf product, post-booster titers were maintained at a satisfactory level for at least five years.

Two purified bivalent Type AB toxoids containing 10.2 Lf of Type A and 16.2 Lf of Type B toxoid per milliliter were also studied in man. Four schedules of immunization were investigated in groups of 25 to 50 individuals with the first bivalent preparation: 0.5-ml doses were given subcutaneously at 0 and 8 weeks; 0 and 10 weeks; 0, 2, and 10 weeks; and 0, 2, 4, and 6 weeks. A 0.5-ml booster dose of toxoid was given one year after the initial injection, except that those with the 0-10 week schedule boosters were given after six months. Serum antitoxin titers of individuals were determined two to three weeks after the initial series and again eight weeks after the booster.

* The Lf value of a toxin is the number of units of antitoxin with which 1.0 ml of toxin flocculates in the shortest interval of time.

The effect of schedule on the primary antitoxin response is shown in Figure 1. The striated bars represent the Type A response and the solid bars represent the Type B response. The graph shows the percentage of persons that had measurable Types A and B titers following initial immunization. Numbers in the bars are the weeks after the initial injection of toxoid.

The percentage of individuals having demonstrable titers following the initial series of injections depended upon the immunization schedule. Injections administered at 0, 4, and 10 weeks produced the greatest responses, and this schedule was selected for routine immunization of man.

Although not shown, marked booster responses were obtained in all cases irrespective of primary immunization schedule, and a 12-month booster appeared to be more effective than a six-month booster. Available data showed that the Type A and Type B titers were maintained at a satisfactory level for at least two years after the 52-week booster. These results indicated that when a rapid, high initial response is not desired an 0-8 or 0-10 schedule with a 52-week booster would be quite satisfactory.

Experiments were then conducted to extend observations on univalent Type A and bivalent Type AB toxoids to pentavalent preparations containing the five purified toxoids in combination with aluminum phosphate. On the basis of exploratory investigations, two combinations of the five toxoids were formulated.

The composition of the first pentavalent preparation, referred to as ABCDE-1, is shown in Table II. Concentrations of Types A, B, and D antigens are expressed in Lf. Porton antitoxins were used for the Lf measurements. The Type E antigen in this toxoid was expressed in terms of Lf, but difficulty was encountered with the flocculation of the purified Type E toxins, and in toxoids 6, 7, and 8 the concentrations of Type E are expressed in terms of LD_{50} of the purified toxin before detoxification. The purified Type C toxin did not flocculate; therefore, the concentrations of all Type C antigens were expressed in terms of LD_{50} equivalents also. Five univalent preparations were also prepared, each of which contained the same concentration of antigens as in ABCDE-1. A second pentavalent preparation, ABCDE-2, contained each toxoid at one-fifth the concentration present in the first. All toxoids contained seven milligrams per ml of aluminum phosphate.

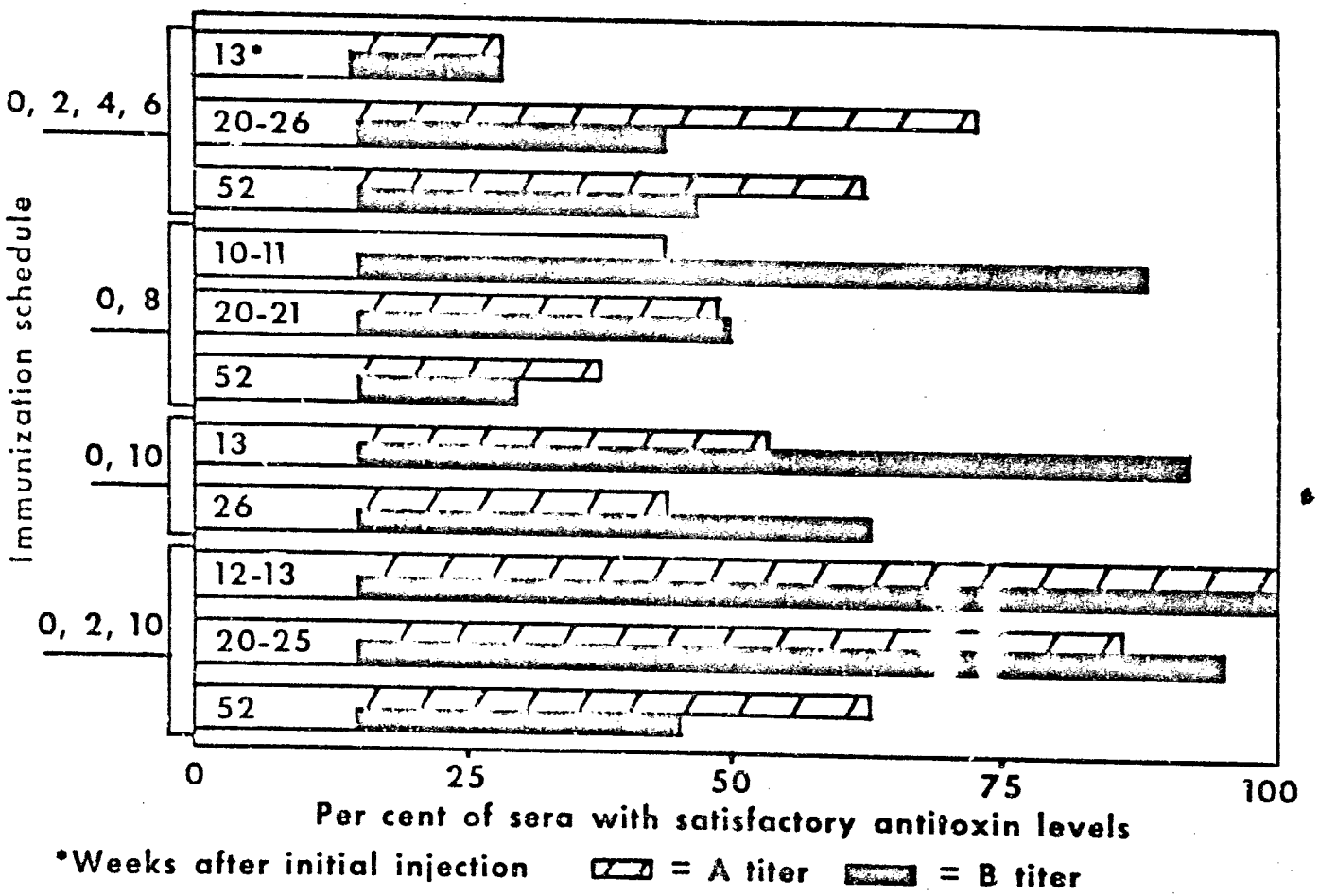

Figure 1. Effect of Schedule of Immunization on Antitoxin Response.

TABLE II. COMPOSITION OF THE PENTAVALENT TOXOIDS

Type	Concentration of Antigen
A	1.7 Lf per ml
B	0.54 Lf per ml
C	50,000 LD_{50} equivalents per ml
D	4.0 Lf per ml
E	5.0 Lf per ml (ABCDE-1) 100,000 LD_{50} equivalents per ml (ABCDE-6, -7, and -8)

Seventeen persons were immunized with the ABCDE-1 toxoid and five or six persons per type were immunized with the univalent control toxoids on a 0-2-10 week schedule. Individual titers were determined 12 and 52 weeks after the initial injection; boosters were given at 52 weeks, and titers were determined eight weeks after the booster injection.

The data obtained 12 weeks after the initial injection and eight weeks after the booster dose are presented in Table III. Antigenic responses to all antigens were found 12 weeks after the initial injection. Though not shown, a considerable drop in titer occurred between 12 and 52 weeks. Fifty-two weeks after the initial injection a small percentage had measurable Types A, B, C, and D titers and 94 per cent had measurable Type E titers. With the administration of a booster all titers rose above the levels that had been obtained at 12 weeks. Eight weeks after the booster one individual immunized with the pentavalent toxoid did not have a measurable Type B titer; all others had titers to all antigens. The median titers for each type ranged from 20 to 640 times the lowest measurable level. The univalent groups were not large enough to make adequate comparison of the univalent and pentavalent response; however, statistical analysis by the rank sum method showed that the individual responses to the pentavalent toxoid were generally not significantly different from the corresponding univalent responses at either the 12-week or the 8-week post-booster bleeding.

TABLE III. ANTITOXIC RESPONSE IN MAN TO PENTAVALENT TOXOID ABCDE-1

Bleeding Time	Type	Per cent with Measurable Titers	Porton Units Per Ml of Serum	
			Median	Range
12 weeks	A	65	0.05	<0.02 - 0.3
	B	82	0.03	<0.005 - 0.2
	C	88	0.2	<0.02 - 1.2
	D	47	<0.16	<0.16 - 1.9
	E	94	0.3	<0.0125 - 2.0
8 weeks post-booster	A	100	0.6	0.03 - 6.4
	B	94	0.1	<0.005 - 1.3
	C	100	1.2	0.1 - 13.3
	D	100	4.0	0.5 - 51.2
	E	100	8.0	0.3 - 80.3

A major objective of our program was to obtain a high percentage of persons with measurable levels of antitoxin after the initial series of injections. Preliminary data indicated that an increase in time between the second and third injection in the initial series would increase the percentage of individuals with measurable titers. Three additional pentavalent toxoids were studied simultaneously.

Groups of 30 persons were immunized with ABCDE-6, -7, and -8 on a 0-2-12 week schedule; a 0.5-ml booster was given 52 weeks after the initial injection. All individuals were bled 14 and 52 weeks after the initial injection and eight weeks after the booster. Antitoxin titers were determined for each type on each serum.

The percentage of individuals exhibiting measurable titers two weeks after completion of the initial series with each of the pentavalent toxoids is presented in Table IV. The data obtained with the ABCDE-1 toxoid are also presented for comparison. Two weeks after completion of the initial series a large proportion of the individuals had titers to all types regardless of the toxoid used. Response to the Type C antigen was excellent in all preparations and high percentages of individuals had Types A, B, and E titers. The poorest response was exhibited with the Type D antigen. The median titers ranged from less than measurable with the Type D to as high as 24 times the measurable level with Type E.

TABLE IV. COMPARATIVE INITIAL RESPONSES IN MAN TO FOUR PENTAVALENT TOXOIDS[a/]

		ABCDE-1	ABCDE-6	ABCDE-7	ABCDE-8
Type A	% Measurable	65	90	97	81
	Median, units/ml	0.05	0.2	0.2	0.03
Type B	% Measurable	82	93	80	59
	Median, units/ml	0.03	0.03	0.02	0.008
Type C	% Measurable	88	100	87	89
	Median, units/ml	0.2	0.3	0.1	0.1
Type D	% Measurable	47	79	60	52
	Median, units/ml	<0.16	0.5	0.5	0.2
Type E	% Measurable	94	100	90	63
	Median, units/ml	0.3	0.2	C.08	0.02

a. Toxoid 1 - 0, 2, 10 week schedule.
Toxoids 6, 7, and 8 - 0, 2, 12 week schedule.

The general trend appeared to be toward an increased response with the 0-2-12 week schedule and this was selected for routine immunization. Although not shown, all titers declined between 14 and 52 weeks and only a small portion of the individuals had measurable titers at 52 weeks.

The data obtained eight weeks after the 52-week booster are shown in Table V. It may be seen that a booster given at 52 weeks was very effective and with toxoids -6, -7, and -8, all except three titers were measurable eight weeks after the booster. The median titers and percentages with measurable titers are summarized here. In all cases the median titers were less than the mean titers; the means, though not shown, ranged from 1.3- to 5.5-fold of the median titers. After the initial series median titers were 2 to 16 times the lowest measurable level; after the booster, they ranged from 10- to 100-fold the measurable level. Responses after the booster were similar to those obtained with toxoid -1.

TABLE V. COMPARATIVE BOOSTER RESPONSES IN MAN TO FOUR PENTAVALENT TOXOIDS

		ABCDE-1	ABCDE-6	ABCDE-7	ABCDE-8
Type A	% Measurable	100	100	100	100
	Median, units/ml	0.6	2.1	2.1	0.8
Type B	% Measurable	94	100	86	94
	Median, units/ml	0.1	0.3	0.2	0.03
Type C	% Measurable	100	100	100	100
	Median, units/ml	1.2	1.2	0.8	0.6
Type D	% Measurable	100	100	89	100
	Median, units/ml	4.8	2.4	1.2	2.0
Type E	% Measurable	100	100	100	100
	Median, units/ml	8.0	1.0	0.2	0.2

IV. EFFICACY OF TOXOIDS IN ANIMALS

Throughout the development and evaluation studies, attention was given to the response of laboratory animals to botulinum toxoids. In animals estimation of the antigenicity of botulinum antigens is based not only on development of serum antitoxin titers, but also on development of resistance to challenge with toxin. Titrations carried out in mice showed the absorbed toxoids to be highly antigenic. Maximum protection to intraperitoneal challenge was obtained three to five weeks after immunization. At this time the immunized animals survived 10^5 to 10^6 mouse LD_{50}. Addition of the aluminum phosphate adjuvant markedly increased the antigenicity of the toxoids for mice. With the Type C toxoid, protective activity of the adsorbed preparations was approximately 30 times as great as that obtained with the non-adsorbed.

Early in our studies consideration was given to the relationship between the serum antitoxin titer and the degree of resistance to botulinum intoxication. Experiments were carried out in guinea pigs, which were immunized subcutaneously with aluminum-phosphate-adsorbed univalent toxoids and challenged intraperitoneally with homologous toxin. Serum samples were obtained the day before challenge for antitoxin determinations. Figure 2 shows the resistance to intraperitoneal challenge as a function of antitoxin titer in guinea pigs. Antitoxin titers are shown on the horizontal axis. Arrows on this axis designate the lowest measurable titer for each type of antitoxin. The 50 per cent survival end point, expressed in $MIPLD_{50}$, is shown on the vertical axis. Note that as antitoxin titer increased, the level of resistance also increased and in several cases appeared to reach a maximum.

Additional studies were carried out in rabbits and guinea pigs to determine the antigenic response to univalent and pentavalent toxoids as a function of type, dose, time, and a booster dose of toxoid.

Separate groups of rabbits and guinea pigs were given subcutaneous injections of the ABCDE-1, and -2 and of the five univalent control toxoids described earlier. A booster dose was given to rabbits after 90 days and to guinea pigs after 180 days. Antitoxin titers were determined at intervals after immunization.

A comparison of the Type B antitoxin response in rabbits to the univalent and pentavalent toxoids is shown in Figure 3 to illustrate the nature of the responses. The maximum response to the Type B antigen in rabbits to a single injection of univalent or pentavalent toxoids was obtained in approximately 50 days. In no case did the response to the B antigen in the pentavalent preparations reach that obtained to the univalent antigen. Response to the B antigen in the -2 toxoid was also lower than the response to this antigen in the -1 toxoid. Titers increased

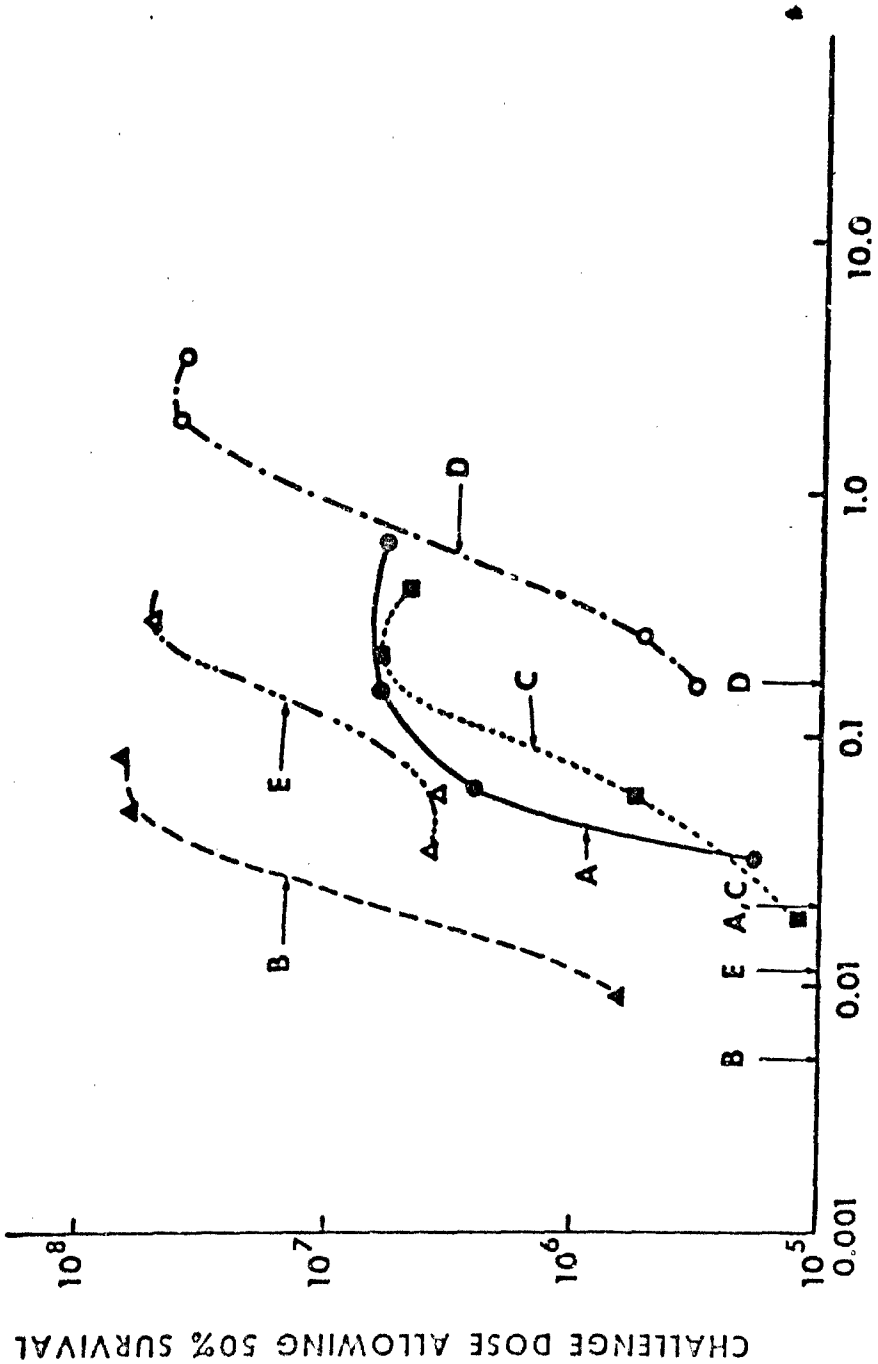

Figure 2. Resistance to Intraperitoneal Challenge as a Function of Serum Antitoxin Titer in Guinea Pigs.

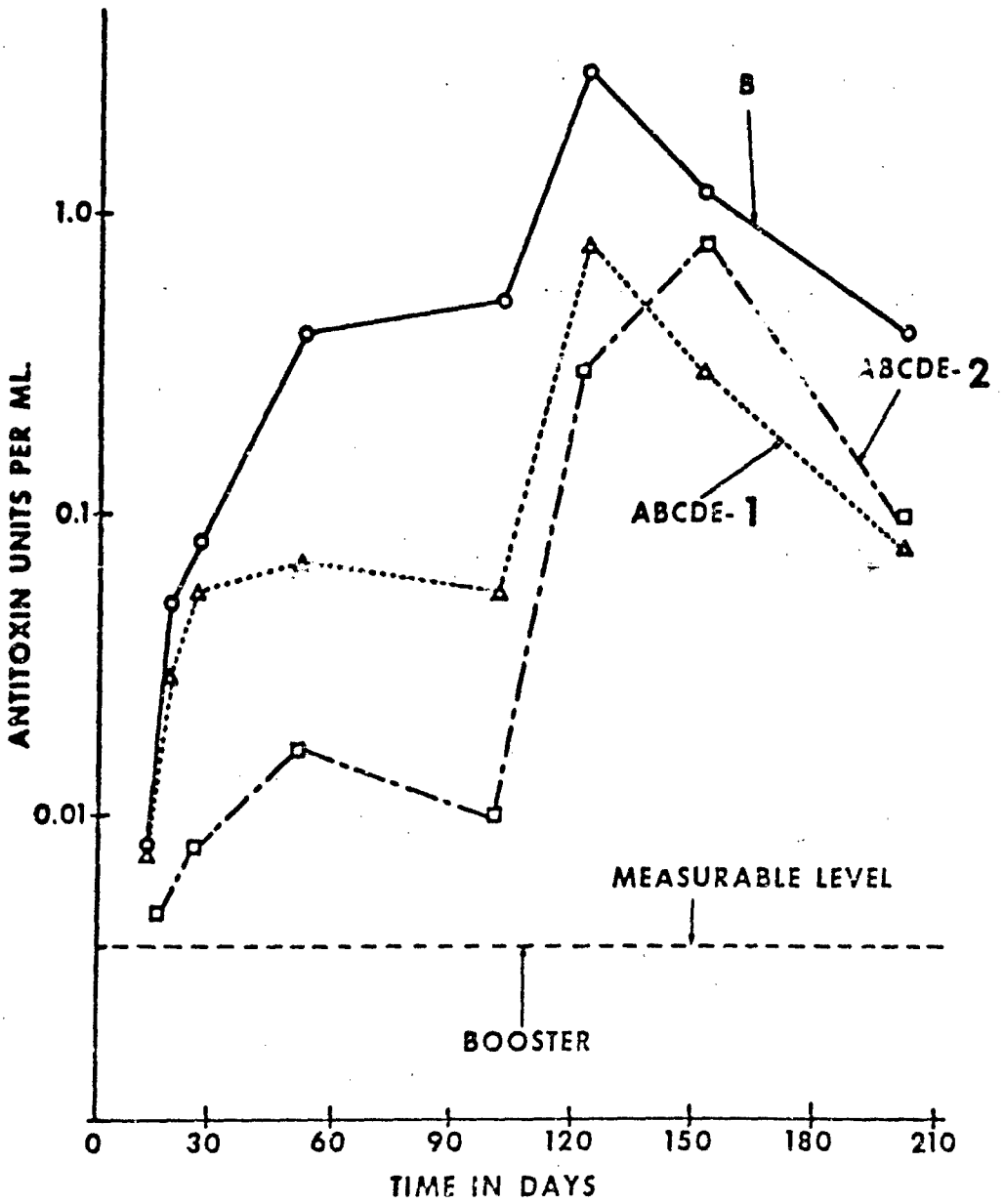

Figure 3. Type B Antitoxin Response in Rabbits.

following the 90-day booster injections. It may be seen that responses paralleled each other, both before and after the booster injections, and that the best booster response was obtained with the ABCDE-2 toxoid. The results obtained in guinea pigs were generally similar. Though not shown, all toxoid components given individually or in combination stimulated satisfactory antitoxin responses following primary immunization, and the titers increased following booster injections. Antibody formation was more rapid with the more concentrated pentavalent toxoid in both rabbits and guinea pigs. The results in both species are summarized in Table VI.

TABLE VI. MAXIMUM INITIAL ANTITOXIN RESPONSE TO ABCDE-1 RELATIVE TO UNIVALENT CONTROLS

Type	Titer with Pentavalent/Titer with Univalent	
	Rabbits	Guinea Pigs
A	10%	140%
B	14%	4%
C	95%	20%
D	33%	14%
E	37%	20%

With few exceptions the results indicate that formation of individual antibodies was decreased when the toxoids were given in a pentavalent combination. The repression appeared to be less marked during the secondary response following booster. In general, a slower rate of antibody formation and a more marked repression were observed in guinea pigs than in rabbits. Data are not available for conversion of the antitoxin units to weight units, and accordingly the total amount of antibody produced in response to the pentavalent combination cannot be compared in absolute terms with the univalent controls. It appears, however, that the total antibody response to the combination was not greatly different from that to the univalent antigen. It seems probable that the decreased individual responses represented a competition of the antigens for the antibody-forming mechanism, or possibly for the adjuvant activity of the aluminum phosphate.

To further evaluate the efficacy of botulinum antigens, consideration was given to the resistance afforded immunized animals to challenge by various routes.

It has been established that inhalation of botulinum toxins in moderate doses is lethal to laboratory animals.[66] It seemed possible that respiratory challenge might provide a more severe test of the efficacy of toxoids than challenge by other routes. Support for this view was provided by Iakolev's[67,68] finding that higher levels of antitoxin were required for prophylaxis against inhalation of botulinum toxin than against other routes. I would like now to present to you results of some of our studies to determine resistance afforded actively immunized guinea pigs to challenge with botulinum toxins by various routes.

The toxins used in our initial studies were produced from cultures grown in cellophane tubing immersed in nutrient medium. Cultures were clarified by centrifugation and used without further concentration or purification, since unaltered cell-free preparations were considered to be most suitable. Type E toxin was activated with trypsin prior to clarification of culture and subsequently stored in the frozen state.

Intraperitoneal and oral LD_{50} estimates of toxins were determined in mice and guinea pigs. Respiratory LD_{50} estimates were determined in guinea pigs exposed bodily to static aerosols of the toxins. More than 60 per cent of the particles in the aerosols were five microns or less in diameter. The guinea pig inhaled dose, in terms of MIPLD$_{50}$ units, was estimated by titration of the collecting fluid from impinger samplers. Serum antitoxin titers of the immunized animals were determined on sera obtained by cardiac bleeding of the animals the day before challenge.

Before immunized guinea pigs were challenged, the toxicities for normal guinea pigs were determined in terms of mouse LD_{50} units and were then translated into guinea pig units. These estimates for the five toxins administered by the various routes are summarized in Table VII. The results are expressed in mouse intraperitoneal LD_{50}. As may be seen, the animals were most susceptible by the intraperitoneal route, as expected, and were more susceptible to toxin administered by the respiratory route than by the oral route.

Expression of the respiratory LD_{50} estimates in terms of guinea pig intraperitoneal LD_{50} revealed that guinea pigs show essentially similar susceptibility to the five toxins administered by the respiratory route. The respiratory susceptibility is more closely related to the oral susceptibility than to their intraperitoneal susceptibility with four of the five types.

TABLE VII. TOXICITY OF BOTULINUM TOXINS FOR THE GUINEA PIG BY VARIOUS ROUTES

Type	Toxicity in MIPLD$_{50}$ Units		
	IP	Respiratory	Oral
A	5.2	141	717
B	4.2	350	306
C	1.6	87	177
D	4.1	186	436
E	34.3	778	178,000

In our first series of experiments, groups of guinea pigs were immunized by the subcutaneous route, each with a single injection of univalent toxoid. Each of the univalently immunized groups of guinea pigs was divided into three subgroups, which were challenged 36 to 40 days after immunization with homologous toxin by the oral, respiratory, and intraperitoneal routes respectively. Normal control groups were challenged in the same manner.

The respiratory challenge results are summarized in Table VIII. Average antitoxin titers and the range of titers obtained for each type of immunization are shown in the second column. The challenge doses are shown in the third column. Note that in this and all subsequent tables the challenge doses are expressed in terms of guinea pig LD_{50} by each route. Challenge results and per cent survivals are also shown. Note that 79 to 91 per cent of the actively immunized guinea pigs survived this level of respiratory challenge. Although not shown in Table VIII, 70 to 100 per cent of the subgroups challenged by the intraperitoneal and oral routes respectively survived challenge of 10^4 to 10^5 LD_{50}.

Average antitoxin titers ranged from 10 to 50 times measurable levels, and although a considerable variation in antitoxin titers was obtained, the high percentage of survivors made it impractical to test for a relationship between serum antitoxin titers of individual animals and resistance to challenge.

TABLE VIII. GUINEA PIG RESPONSE TO AEROSOL CHALLENGE WITH BOTULINUM TOXINS

Toxin Type	Antitoxin Titer	Respiratory Challenge, LD_{50} units	No. Survivors Total/Exposed	Per Cent Survival
A	0.3 <0.04-0.96	5	40/44	91
B	0.059 <0.005-0.8	7	30/38	79
C	0.4 <0.02-1.9	8	38/44	86
D	6.1 <0.32-21	9	35/39	90
E	0.29 <0.025-2.0	3	26/32	81

It appeared evident from these results that higher levels of challenge would be necessary to determine the magnitude of resistance to respiratory challenge. A second test in this series of experiments was designed to accomplish this.

Groups of guinea pigs were immunized with either univalent Type D or pentavalent botulinum toxoids and subsequently challenged with graded doses of toxin by the respiratory route. Type D was selected because the toxicity of the culture permitted higher challenge doses. Immunization with pentavalent toxoid was included so that effectiveness of an antigen in a multivalent preparation and a univalent antigen could be compared. Results of this test are shown in Table IX.

The type of immunization is shown in the first column. One-half milliliter was employed as an immunizing dose for the univalent group and for the first pentavalent group, and these groups were challenged 40 days after immunization. The second pentavalent group received 1.0 milliliter as an immunizing dose and was challenged 50 days after immunization.

Subgroups were challenged with graded doses of Type D toxin by the respiratory route. Smaller groups of guinea pigs immunized in the same manner were challenged by the intraperitoneal and oral routes.

TABLE IX. CHALLENGE OF IMMUNIZED GUINEA PIGS WITH TYPE D TOXIN BY THE RESPIRATORY ROUTE

Type of Immunization	Antitoxin Titer	Respiratory Challenge, LD_{50} units	No. Survivors Total/Exposed	Per Cent Survival
	3.8	22	40/40	100
Univalent		162	40/40	100
(0.5 ml)	3.2-5.1	2100	40/40	100
	0.63	26	20/20	100
Pentavalent		277	20/20	100
(0.5 ml)	<0.16-1.9	2090	16/20	80
		26	20/20	100
Pentavalent	1.5	230	20/20	100
(1.0 ml)	0.32-5.8	1980	20/20	100
None	-	1	6/20	30

The second column shows the average antitoxin titers of serum pools obtained the day before challenge and the range of titers obtained on these pools. Challenge results and per cent survivals are shown in the next columns. Essentially all of the actively immunized guinea pigs survived 20 to 2000 respiratory LD_{50}. Although not shown in Table IX, 60 to 100 per cent of comparable immunization groups survived 10^6 guinea pig LD_{50} when challenged either by the intraperitoneal or by the oral route.

Additional experiments were conducted with dynamic aerosols generated in the Henderson apparatus. Groups of guinea pigs were immunized with a 0.5-ml dose of pentavalent toxoid and challenged after 40 days with a single type of toxin by the intraperitoneal, oral, and respiratory routes.

Table X presents a summary of the results obtained with the Types A and D challenges. The immunization of guinea pigs with pentavalent toxoid afforded a high level of protection to challenge by the three challenge routes employed. Resistance to challenge by the respiratory route was similar to resistance to challenge by the intraperitoneal and oral routes. Essentially similar results were obtained with the Types B, C, and E toxins.

TABLE X. RESISTANCE AFFORDED PENTAVALENTLY IMMUNIZED GUINEA PIGS TO CHALLENGE BY VARIOUS ROUTES

Toxin Challenge			Challenge Results	
Type	Route	LD_{50}	Survivors/Total	Per Cent Survival
A	Intraperitoneal	3×10^8	8/15	53
	Oral	5×10^8	15/16	94
	Respiratory	3×10^4	11/12	92
D	Intraperitoneal	2×10^8	8/14	57
	Oral	4×10^8	8/10	80
	Respiratory	2×10^4	6/13	46

V. DISCUSSION

Dr. Murray: Thank you very much. This paper is now open for discussion and comments. It certainly has indicated the possibilities of active immunization and much of the data presented, at least in the case of animals, would indicate the relationship of the antibody levels that must be present to protect against the different amounts of toxin given by different routes. I think this, in itself, is important information.

Q. Dr. Rogers - Nashville. Mr. Cardella, I am very impressed with the total response. Did you do any studies on the incidence of antibody response after the first, second, and third shot of toxoid in your studies?

A. With regard to the pentavalent immunizations, we have not. However, in a small group of individuals antitoxin titers were determined following immunization with the univalent and the bivalent antigens. There were indications that a very small percentage developed antitoxin titer after a single injection of a more concentrated toxoid. A higher percentage had titers after the second and, of course, as described, after the third injection or after a fourth. A higher proportion has measurable titers after the series and this, as I described, was the basis for spacing the injections to actually take advantage of what appeared to be anamnestic reactions.

Q. From the audience: Mr. Cardella, my question is - how did you establish the dose that you applied in the inhalation challenge?

A. This was done by first determining the breathing rates and volumes of the animals exposed to the aerosols. During animal exposure, concentration of toxin in the aerosols was determined by obtaining air samples in appropriate pre-rated air samplers containing collecting fluid. This fluid was titrated for toxin concentration. Estimates of animal dose inhaled were made from the data obtained based upon the relationship between concentration of toxin per volume of air sampled and volume of air inhaled by the animals.

Q. From the audience: Could you say something about possible reactions to the toxoid?

A. We have seen what we consider to be mild to moderate local reactions. I think some of you may have observed this. It has been described as a small nodule of varying size that persists for varying periods of time. Incidence of this with the pentavalent toxoid is about one per cent. Approximately 1000 individuals have received this toxoid. Indications are that the percentage of reactions to the pentavalent may be slightly higher

than to our univalent or bivalent preparations. This may be due to the adjuvant, which has been modified slightly. We are now using a Holt's seven-eighth aluminum phosphate gel, which is essentially seven-eighths aluminum phosphate and one-eighth aluminum hydroxide. The previous phosphate was essentially all ortho-phosphate. I might point out that in our pentavalent preparations the Types C, D, and E antigens were not taken to the degree of purity that the A and B were. It is possible that inclusion of one of these antigens might account for the slightly higher reaction rate. It appears to be at a relatively low rate, however.

Q. I am Dr. Petty from Baltimore, Mr. Cardella. I wanted to thank you for a very interesting and very beautifully presented paper that interested most of us here. I have one question. Did you see any total failures of response in humans to the pentavalent toxoid and, if so, can these be explained in any manner and, secondly and part of this same question, would you consider these people resistant to botulinum toxin?

A. We have seen lack of response to any antigen in the primary immunizations. We have followed approximately 120 individuals, and four of these showed no response to any of the five antigens after the initial series. I might point out that approximately 41 per cent showed titer to all five antigens and at least 79 per cent showed measurable titer to at least four of the five antigens. The incidence of no measurable response was fairly low.

Failure to demonstrate antitoxin titers following toxoid injections could be attributed to the limit of sensitivity of the neutralization test employed to measure titers. With regard to considering these people resistant to botulinum toxin, it can be pointed out that laboratory animals with no demonstrable antitoxin titers following an injection of toxoid were resistant to toxin. In fact, the apparent non-responder may have produced antitoxin that is not measurable by our test. For the absolute non-responder I would not recommend a willful exposure to toxin.

LITERATURE CITED

1. Meyer, K.F. "The status of botulism as a world health problem," Bull. World Health Organ. 15:281-289, 1956.

2. Dolman, C.E. "Human botulism and staphylococcal food poisoning: A review," Japan J. Med. Sci. Biol. 10(6):373-381, 1957.

3. Weinberg, M., and Goy, P. "Recherches sur la toxine botulinque," Compt. Rend. Soc. Biol. 90:269-271, 1924; "De l'anatoxine botulinque," Ibid. 91:148-149, 1924; "Etude comparative des toxines botulinques formolee et iodee," Ibid. 91:1140-1141, 1924; and "Emploi. de l'anatoxine dans la preparation du serum antibotulique," Ibid. 92:564-565, 1924.

4. Graham, R.; Tunnicliff, E.A.; McCulloch, E.C.; and Thorp, F., Jr. "The antigenic value of formalized botulinum toxins," J. Am. Vet. Med. Assoc. 75:21-37, 1929.

5. Graham, R., and Thorp, F., Jr. "Antigenic value of botulinum toxoids kept one year at ice box temperature," J. Immunol. 20:305-312, 1931.

6. Velikanov, I.M. "Experimental vaccination against botulism," Immunitaetsforsch. 70:186-194, 1931.

7. Legroux, R., and Jeramec, C. "Etudes sur la toxine et l'antitoxine botuliques," Compt. Rend. Soc. Biol. 120:651-643, 1935.

8. Jakobkiewicz, J. "Toxine'et anatoxine botulique," Compt. Rend. Soc. Biol. 123:262-264, 1936.

9. Jeramec, C. "Toxine of anatoxine botulique," Rev. Immunol. 2:209-220, 1936.

10. Velikanov, I. "Experimental immunization of man against botulism," Klinich. Med. 12:1802-1806, 1934.

11. Velikanov, I. "Immunization experimentale del'homme contre la botulisme," Giorn. batteriol. e immunol. 17:451-456, 1936.

12. Melnik, M.I., and Starobinetz, G.M. "Active immunization against botulism," Ann. Inst. Metch. 4:33-42, 1936.

13. Bennetts, H.W., and Hall, H.T.B. "Botulism of sheep and cattle in western Australia, its cause and its prevention by immunization," Australian Vet. J. 14:105-118, 1938.

14. Mason, J.H.; Steyn, H.P.; and Bisschop, J.H.R. "The immunization of bovines against lamsiekte," J. S. African Vet. Med. Assoc. 9(2): 65-70, 1938.

15. Sterne, M., and Wentzel, L.M. "A new method for the large-scale production of high-titre botulinum formal-toxoid Types C and D," J. Immunol. 65:175-183, 1950.

16. Nigg, C.; Hottle, G.A.; Coriell, L.L.; Rosenwald, A.S.; and Beveridge, G.W. "Studies on botulinum toxoid, Types A and B: I. Production of alum-precipitated toxoids," J. Immunol. 55:245-254, 1947.

17. Hottle, G.A.; Nigg, C.; and Lichty, J.A. "Studies on botulinum toxoids, Types A and B: II. Methods for determining antigenicity in animals," J. Immunol. 55:255-262, 1947.

18. Rice, C.E.; Pallister, E.F.; Smith, L.C.; and Reed, G.B. "Clostridium botulinum Type A toxoids," Can. J. Research Sect. E 25:167-174, 1947.

19. Rice, C.E.; Smith, L.C.; Pallister, E.F.; and Reed, G.B. "Clostridium botulinum Type B toxoids," Can. J. Research Sect. E 25:175-180, 1947.

20. Rice, C.E. "Preliminary study of antigenic activity of mixtures of Clostridium botulinum toxoid Types A and B," Can. J. Research Sect. E 25:181-187, 1947.

21. Prevot, A.R., and Brygoo, E.R. "Recherches sur la toxine, l'anatoxine et l'antitoxine botuliques D," Ann. Inst. Pasteur 79:1-13, 1950.

22. Moberg, K. "Ueber die Festellung and Bekämpfung des Botulismus beim Nerz in Schweden," Proc. XV Intern. Vet. Congr. (Stockholm), 1:62-64, 1953.

23. Dinter, Z. "Active immunity against Cl. botulinum Type C toxin," Nord. Veterinarmed. 9:1056-1062, 1955.

24. Larsen, A.E.; Nicholes, P.S.; and Gebhardt, L.P. "Successful immunization of mink with a toxoid against Clostridium botulinum, Type C," Am. J. Vet. Res. 16(61):573-575, 1955.

25. Appel, R.N.; Hartsough, G.R.; McCoy, E.F.; and Brandly, C.A. "Active and passive protection of mink against type C botulism with type C toxoid and polyvalent antitoxin," J. Am. Vet. Med. Assoc. 128(11):556-558, 1956.

26. Draeger, K.; Schindler, R.; and Vocke, G. "Experimental immunization of mink against distemper and botulism," Tieraerztl. Umschau. 12:136-138, 1957.

27. Pitre, J., and Quittet, P.L. "Immunizing power of vaccine against Clostridium botulinum type C in mink," Bull. Acad. Vet. France 31: 379-382, 1958.

28. Matveev, K.I.; Bulatova, T.I.; and Sergeeva, T.I. "Immunization of minks against botulism," Veterinariya 35(8):42-46, 1958.

29. Appleton, G.S., and White, P.G. "Field evaluation of Clostridium botulinum type C toxoids in mink," Am. J. Vet. Res. 22(74):166-169, 1959.

30. Prevot, A.R., and Jilliot, R. "Studies of botulism in mink and ferrets and vaccination against botulism," Bull. Acad. Vet. France 30:93-98, 1957.

31. Pridham, T.J. "Simultaneous immunization of mink against virus enteritis, distemper and botulism," Can. Vet. J. 2:212-216, 1961.

32. Matveev, K.I.; Bulatova, T.I.; and Sergeeva, T.I. "Large-scale immunization of mink against botulism," J. Microbiol. Epidemiol. Immunobiol. 32(11):138-140, 1961.

33. Boroff, D.A., and Reilly, J.R. "Studies of the toxin of Clostridium botulinum: V. Prophylactic immunization of pheasants and ducks against avian botulism," J. Bacteriol. 77(2):142-146, 1959.

34. Boroff, D.A., and Reilly, J.R. "Immunization of pheasants against the toxin of Clostridium botulinum Type C," Bacteriol. Proc. 60:89, 1960.

35. Boroff, D.A., and Reilly, J.R. "Studies on the toxin of Clostridium botulinum: VI. Botulism among pheasants and quail, mode of transmission, and degree of resistance offered by immunization," Intern. Arch. Allergy and Appl. Immunol. 20:306-313, 1962.

36. Schwartz, L.K. "Control of botulism in wild fowl," J. Am. Vet. Med. Assoc. 143:163, 1963.

37. Barron, A.L., and Reed, G.... "Clostridium botulinum Type E toxin and toxoid," Can. J. Microbiol. 1(2):108-117, 1954. Also Vet. Bull. 25:2321, 1955.

38. Gunnison, J.B.; Cummings, J.R.; and Meyer, K.F. "Clostridium botulinum Type E," Proc. Soc. Exptl. Biol. Med. 35:278-280, 1936.

39. "Collection of references and annotations of scientific works of the institute for 1913-1929," Kushnir, E.D. ed. Dnepropetrovsk Sanitary-Bacteriological Institute, Dnepropetrovsk, 1940. p. 14.

40. Hazen, E.L. "Differential characteristics of two strains of Cl. botulinum, Type E," Proc. Soc. Exptl. Biol. Med. 50:112-114, 1942.

41. Dolman, C.E.; Chang, H.; and Karr, D.E. "Fish-borne Type E botulism: Two cases due to home pickled herring," Can. J. Pub. Health 41:215-229, 1950.

42. Prevot, A.R., and Huet, M. "Existence en France du botulisme humain d'origine pisciare et de Cl. Botulinum E," Bull. Acad. Natl. Med. 25 and 26:432, 435, 1951.

43. Prevot, A.R., and Brygoo, E.R. "Recent research on botulism and the toxin types of Clostridium botulinum," Ann. Inst. Pasteur 85:544-575, 1954. Also Bull. Hyg. 29(5):528-529, 1954.

44. Koroleva, G.A.; Matveev, K.I.; and Volkova, Z.M. "The preparation of therapeutic Types C and E botulinum antisera in horses: Part 1," J. Microbiol. Epidemiol. Immunobiol., 29(3)102-106, 1958. Part 2, Ibid. 29(5)83-85, 1958.

45. Kravchenko, A.T., and Rezepov, F.F. "Preparation of Clostridium botulinum antisera in cattle," J. Microbiol. Epidemiol. Immunobiol. 30(4):79-82, 1959.

46. Rezepov, F.F. "The experimental effectiveness of Types C and E concentrated antibotulism sera," J. Microbiol. Epidemiol. Immunobiol. 31(4):87-93, 1960.

47. Chertkova, F.A.; Grodko, N.S.; Ushakova, A.A.; Denisova, I.A.; Kats, F.M.; and Dudarenko, G.V. "Standard Clostridium botulinum Type E antiserum," J. Microbiol. Epidemiol. Immunobiol. 31(4):682-685, 1960.

48. Dolman, C.E., and Murakami, L. "Clostridium botulinum Type F with recent observations on other types," J. Infect. Diseases 109:107-128, 1961.

49. Bowmer, E.J. "Antitoxins of Clostridium botulinum Types A, B, C, D, and E: Preparation and assay of proposed international standards," A thesis submitted for the Degree of M. D., Faculty of Medicine, University of Liverpool, April, 1962.

50. Dolman, C.E., and Iida, H. "Type E botulism: Its epidemiology, prevention, and specific treatment," Can. J. Pub. Health 54:293-308, 1963.

51. Reams, H.R.; Kadull, P.J.; Housewright, R.D.; and Wilson, J.B. "Studies on botulinum toxoid, Types A and B: Immunization of man," J. Immunol. 55:309-324, 1947.

52. Duff, J.T.; Wright, G.G.; Klerer, J.; Moore, D.E.; and Bibler, R.H. "Studies on immunity to toxins of Clostridium botulinum: I. A simplified procedure for isolation of Type A toxin," J. Bacteriol. 73:42-47, 1957.

53. Duff, J.T.; Klerer, J.; Bibler, R.H.; Moore, D.E.; Gottfried, C.; and Wright, G.G. "Studies on immunity to toxins of Clostridium botulinum: II. Production and purification of Type B toxin for toxoid," J. Bacteriol. 73:597-601, 1957.

54. Duff, J.T.; Wright, G.G.; and Yarinsky, A. "Activation of Clostridium botulinum Type E toxin by trypsin," J. Bacteriol. 72(4):455-460, 1956.

55. Wright, G.G.; Duff, J.T.; Flock, M.A.; Devlin, H.B.; and Soderstrom, R.L. "Studies on immunity to toxins of Clostridium botulinum: V. Detoxification of purified Type A and Type B toxins, and the antigenicity of univalent and bivalent aluminum phosphate adsorbed toxoids," J. Immunol. 84:384, 1960.

56. Gordon, M.; Flock, M.A.; Yarinsky, A.; and Duff, J.T. "Studies on immunity to toxins of Clostridium botulinum: III. Preparation, purification, and detoxification of Type E toxin," J. Bacteriol. 74:533-538, 1957.

57. Flock, M.A.; Yarinsky, A.; and Duff, J.T. "Studies on immunity to toxins of Clostridium botulinum: VII. Purification and detoxification of trypsin-activated Type E toxin," J. Bacteriol. 82:66-71, 1961.

58. Flock, M.A.; Devine, L.F.; Gearinger, N.F.; Duff, J.T.; Wright, G.G.; and Kadull, P.J. "Studies on immunity to toxins of Clostridium botulinum: VIII. Immunological response of man to purified bivalent AB botulinum toxoid," J. Immunol. 88:277, 1962.

59. Flock, M.A.; Cardella, M.A.; and Gearinger, N.F. "Studies on immunity to toxins of Clostridium botulinum: IX. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid," J. Immunol. 90:697-702, 1963.

60. Cardella, M.A.; Duff, J.T.; Gottfried, C.; and Begel, J.S. "Studies on immunity to toxins of Clostridium botulinum: IV. Production and purification of Type C toxin for conversion to toxoid," J. Bacteriol. 75:360-365, 1958.

61. Cardella, M.A.; Duff, J.T.; Wingfield, B.H.; and Gottfried, C. "Studies on immunity to toxins of Clostridium botulinum: VI. Purification and detoxification of Type D toxin and the immunologic response to toxoid," J. Bacteriol. 79:372-378, 1960.

62. Cardella, M.A.; Flock, M.A.; and Wright, G.G. "Immunologic response of animals to purified pentavalent ABCDE botulinum toxoid," Bacteriol. Proc. 78, 1958.

63. Cardella, M.A.; Jemski, J.V.; Tonik, E.J.; and Flock, M.A. "Resistance of guinea pigs immunized with botulinum toxoids to aerogenic challenge with toxins," Bacteriol. Proc. 70, 1963.

64. Holt, L.B. "Developments in Diphtheria Prophylaxis," William Heinemann Medical Books, Ltd., London, 1950.

65. Report of Expert Committee on Biological Standardization, World Health Organization, Geneva. (Tech. Rpt Series 259:25, 1963)

66. Lamanna, C. "Immunological aspects of airborne infection: Some general considerations of response to inhalation of toxins," Bacteriol. Rev. 25(3):323-330, 1961.

67. Iakolev, A.M. "The importance of antitoxin immunity for the defense of the body in respiratory penetration of bacterial toxins: I. The role of passive immunity in the defense of the body against respiratory disease caused by Clostridium botulinum toxins," J. Microbiol. Epidemiol. Immunobiol. (USSR) 29:904-909, 1956.

68. Iakolev, A.M. "The importance of antitoxin immunity for the defense of the body in respiratory penetration by bacterial toxins: II. Tests of antitoxin immunity in white mice and guinea pigs upon intranasal administration of Clostridium botulinum toxins," J. Microbiol. Epidemiol. Immunobiol. (USSR) 29:1068-1072, 1956.